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Failure to Elicit the Galli-Mainini Reaction in *Rana pipiens* with Spawning Reflex Fractions and Other Teleostean Pituitary Preparations, and Observations on the Response to Mammalian Gonadotrophins

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Introduction

THE ROLE of male Salientia in human pregnancy diagnosis has received much attention in the medical and clinical literature since its introduction by Galli-Mainini (1947). The test is based on the observation that chorionic gonadotrophin, which is usually present in high concentrations in the urine of women in the first trimester of pregnancy, will cause the release of spermatozoa (the gametokinetic response) in tailless amphibians. Since this response can be induced in frogs in which the anterior lobe of the pituitary has been removed, it is concluded that the mammalian gonadotrophic hormones act directly upon the test animal's gonadal tissue (Kissen, 1954).

The South American toad, *Bufo arenarum,* was the first species to be utilized in pregnancy testing, but since then many different frogs and toads from all over the world have been found suitable. In North America, Wiltberger & Miller (1948) found the readily available and easily maintained leopard frog, *Rana pipiens,* to be both sensitive and reliable. The conditions under which this species may be utilized have been studied by many investigators. During the summer months the leopard frog is least sensitive (Holyoke & Hoag, 1951; Pollak, 1950; Reinhart *et al.,* 1951). Giltz & Miller (1950) showed that leopard frogs kept at cool temperatures and in shallow water were more sensitive than those stored similarly at room temperatures; the optimum temperature for the response, according to these authors, ranges from 15° to 22° C., and reactivity was reported to decrease above and below this. Soucy (1949) found that the reactivity of *R. pipiens* is not affected by keeping these frogs in darkness. The investigations of Haskins & Sherman (1949, 1952) showed that the time of appearance of spermatozoa in the frog's urine is inversely proportional to the amount of chorionic gonadotrophin administered and that this relationship provides a fairly accurate method of bio-assay. Similarly, using pregnancy urine in various dilutions, Marsters *et al.* (1950) found that the intensity as well as the time of the response was related to the hormone titer.

Male amphibians have also been reported to react positively to the pituitary gonadotrophins of mammals (see review by Houssay, 1949). Robbins (1951) and Robbins & Parker (1952) found that follicle stimulating hormone (FSH) caused the release of spermatozoa in *Rana pipiens,* and Hobson (1952) reported that male *Xenopus laevis* are very sensitive to this hormone. On the other hand, Thorborg & Hansen (1951) found that *Bufo bufo* gave no response to FSH in concentrations equivalent to eight Mouse Units. Crézé (1949) reported that the luteinizing hormone (LH) caused the discharge of sperm in *Rana esculenta.* Both FSH and LH gave positive reactions in the Indian toad, *Bufo melanostictus* (Bhaduri, 1951). According to Houssay (1949), *Bufo arenarum,* reacted positively to toad, rat and human hypophysis. Greenblatt, Clark & West (1949) noted that prolactin evoked the gametokinetic reaction in *Rana pipiens,* but Houssay, Penhos & Burgos (1953) found that *Bufo arenarum* did not react to prolactin. No other anterior pituitary hormones have been reported to give a positive gametokinetic response in male Salientia (Robbins & Parker, 1952; Houssay, 1952) and, at the time of writing, the situation in respect to the two gonadotrophins (FSH, LH) and prolactin requires further clarification. Adrenalin is known to elicit this response in frogs but not in toads (Robbins & Parker, 1949; Hinglais & Hinglais, 1949, 1953); however, an error resulting from

this is unlikely under ordinary circumstances and may be discounted.

The present investigation is part of an analysis of the pituitary hormones of fishes undertaken by one of us (G. E. P.) in collaboration with Dr. Alfred E. Wilhelmi of Emory University. It was found (Pickford, 1952) that crude extracts of fish pituitary, as well as certain purified fractions contained a factor that elicited the spawning reflex in both normal and hypophysectomized killifish (*Fundulus heteroclitus*). The problem arose as to whether this factor could be identified with either of the well known mammalian gonadotrophins. Mammalian follicle stimulating hormone (FSH) was found to be inactive, as was also prolactin, but the luteinizing hormone (LH) elicited a moderately strong response. For this reason it was thought that the spawning reflex factor might be identical with LH. The Galli-Mainini test appeared to provide a sensitive and reliable method by which this hypothesis could be tested.

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Materials and Methods

Male frogs, *Rana pipiens,* averaging 30 grms. in weight, were procured from Mr. Walter Daniels, Mt. Ephraim, New Jersey. The animals were housed in a dark, unheated room. The temperature approximated that of the outdoors, and ranged from 5° to 12° C. The well known seasonal variation in the sensitivity of *Rana pipiens* to gonadotrophins cannot be a factor in the present study since the experiments were conducted in winter months, from December, 1953, to middle of March. The frogs were housed in 15-gallon glass aquaria, 10 to 18 animals per tank, with about an inch of tap water covering the slate bottom. Since the animals were not fed, it was sufficient to clean the tanks every five or six days, at which time the frogs were also washed in cold running tap water.

Although most of the animals were in excellent condition, a few showed early symptoms of red leg disease, i.e., a slight reddening of the lower abdomen and legs. Such animals were isolated and treated with chloromycetin in the water, as recommended by Ambrus *et al.* (1951). As a prophylactic measure, chloromycetin was also added to the stock tanks. The incidence of the infection was greatly reduced by this procedure, but the disease was not wholly eradicated.

A few hours before running a test, the heat was turned on in the laboratory, and the temperature raised to 15° to 22° C. This is the optimum range for the gametokinetic reaction (Giltz & Miller, 1950). Frogs to be used for the tests were first examined for the presence or absence of spermatozoa by inserting into the cloaca a thin glass pipette containing a little water. A few drops of cloacal fluid were removed to a glass slide and studied under the microscope. No cases of spontaneous emission were encountered. Test frogs were kept singly in plastic containers, about five inches square with perforated lids; about 2 cc. of water were placed in the bottom of each container. At least two frogs were employed for every test. Animals that gave a positive reaction were kept separately and were not used again for at least one week.

Injections were made into the dorsal lymph sac, using a half-inch, 25-gauge needle. The preparations to be injected were made up in physiological saline (0.7% sodium chloride); concentrations were varied but not the volume injected, which was 1 cc. per frog. The mammalian chorionic gonadotrophin, Antuitrin S (Parke Davis Co.), was used as a standard of reference. Under the conditions of our experiments it was found that 10 I. U., corresponding to 0.04 mgms. of the preparation used (Lot No. M718M), was the minimum dose that would elicit a response within one hour. With this

concentration, occasional or small numbers (up to ca. 75) of motile spermatozoa were seen in most fields under the low power. This order of sensitivity is higher than that reported by Haskins & Sherman (1949), but is of the same order as that found by Holyoke & Hoag (1951). For the purpose of screening the preparations to be tested, a high dosage level was selected, viz. 4 mgms. per cc. per injection; this corresponds to the maximum dosage level that had been used on *Fundulus heteroclitus* in screening the same preparations for spawning reflex activity, viz. 100 microgrs. per gram weight. It is to be noted that this dose is 100 times stronger than the minimum dose of Antuitrin S that was just sufficient to elicit a consistent response. In some cases multiples or fractions of the standard dose were also tested. In testing unknown preparations, two hours were allowed to elapse before the urine was examined.

The following mammalian pituitary preparations were tested: prolactin (Schering Corp., Batch 4g. Hyex 4), luteinizing hormone (Armour Co.), follicle stimulating hormone (Armour Co.), and three preparations submitted by Dr. Wilhelmi. The LH was known to have spawning reflex activity in *Fundulus*, while the prolactin and FSH were inactive in this respect (Pickford, 1952). Two of Dr. Wilhelmi's preparations (B88C and B88E) also had spawning reflex activity, but the third was inactive (P88F). We are indebted to Dr. Sanford L. Steelman for the following additional information regarding the two Armour preparations: "Sheep LH, Lot No. 227-80: This is a purified LH preparation which is about 80 to 85% homogeneous, electrophoretically and ultracentrifugally. It contains traces of FSH and TSH. . . . Swine FSH, Lot No. K45208R: This preparation is a purified FSH which can be briefly characterized by Step II FSH described in Steelman *et al.*, 1953." The last mentioned reference is of particular interest since one may deduce from it that the FSH contained LH activity of the order of three to four percent. The significance of this will appear below.

The fish pituitaries were collected in the summer of 1950 and again in the summer of 1952, from three species of Gadidae: the Boston hake, *Urophycis tenuis* (Mitchill), the cod, *Gadus morhua* Linnaeus, and the pollock, *Pollachius virens* (Linnaeus). Details of the procedure have been described by Pickford (1954). The 1950 batch consisted mostly of pollock, the 1952 batch mostly of hake. This difference has a possible significance, apart from problems of species specificity, since the hake had already spawned whereas the cod and pollock were in pre-spawning condition. The three species were mixed in the bulk material that was frozen and shipped to Dr. Wilhelmi, but a limited number of glands from each species were dropped into acetone, and pulverized powders derived from this material were used in some of the experiments in an attempt to determine whether there were differences between the hake and the other two species. In addition, hake glands were frozen in small packets of ten glands each, and a brei prepared from this material was used in one series of frog tests. Dr. Wilhelmi prepared twelve separate fractions from the 1950 material, all of which were tested on hypophysectomized killifish for anterior lobe activities (Pickford, unpublished). One fraction was toxic, probably owing to the presence of zinc (F7A), four gave evidence of ACTH activity (F4A, F4B, F4C and F5A), three had spawning reflex activity (F6D, F6EF and F6G), one was the fish growth hormone (F6B), and two were inactive (F6C, F31A). Six fractions from the 1952 material were also tested; these had not been subjected to the complete screening survey, as described above, but two of them had moderately strong spawning reflex activity (F80D and F80x), whereas the other four showed little or no activity (Pickford, unpublished). All the fish pituitary preparations, crude or purified, contained a melanophore concentrating agent that induced pallor in killfish (but not in frogs).

Results

(a) *Mammalian Pituitary Preparations.*—Armour's LH was found to have about the same order of activity as Antuitrin S, i.e., a weakly positive response could usually be elicited at a concentration of 0.04 mgms. per cc. Concentrations of 0.1 mgms. per cc. and higher always elicited a vigorous response. Both these preparations were between 40 and 100 times more active than Armour's FSH. The latter preparation was inactive at 2 mgms. per cc. or less, but induced a weak to moderate response at 4 mgms. per cc. The results can be interpreted if it is assumed that the response is elicited by LH but not by FSH, since the FSH preparation used was known to contain three to four percent LH. This order of contamination would account very closely for our results. It would be necessary to use an even more highly purified FSH preparation to determine whether or not this gonadotrophin of itself can evoke the gametokinetic response.

Prolactin was inactive, as also were the three mammalian preparations submitted by Dr. Wilhelmi.

(b) *Fish Pituitary Preparations.*—Of the 20 different fish pituitary preparations that were tested, only the brei of frozen hake glands gave any indications of activity. The response was of a very low order: 3 out of 7 frogs that received an injection of two hake glands per cc. (approximately equivalent to 4 mgms. of dry acetone powder) showed a weak to moderate reaction, the other 4 frogs being negative. Moreover, negative results were obtained at both higher and lower dosage levels: 12 glands (3 frogs), 6 glands (3 frogs), and 0.5 gland (2 frogs). Extracts of an acetone powder of hake glands were completely negative both at 12 and 4 mgms. per cc. (2 frogs each). To make perfectly sure that an occasional positive response might not be obtained, the test on the acetone powder of hake glands was repeated at the 4 mgm. per cc. dosage level, using 10 frogs. The results were again negative. Negative results were also obtained with acetone powders of pollock (12 mgms. per cc. on 2 frogs) and cod (12 mgms. per cc. on 2 frogs; 4 mgms. per cc. on 2 frogs). This finding eliminates the possibility that a seasonal inactivity of the hake glands was involved, resulting from their post-spawning condition.

In view of the findings reported above with crude extracts, it is not surprising that all of the purified or partially purified fish pituitary fractions that were tested also gave negative results. Whatever may be the cause of the weak response that was obtained from the brei of frozen glands, it is clear that there is no correlation with the spawning reflex activity of the preparations.

Discussion

Three separate problems are involved in this investigation: (1) the question as to which mammalian anterior lobe hormone can elicit the Galli-Mainini response, (2) the question as to whether the spawning reflex factor, present in both mammalian and fish pituitary preparations, can be identified with any of the known anterior lobe hormones, and (3) the problem of the reactivity of amphibians to gonadotrophins derived from fishes.

In regard to the first problem, our results suggest that only LH can elicit the response. Previous reports of positive responses with FSH or prolactin can probably be attributed to traces of contamination with LH, as appears to have been the case in the FSH sample which was studied by us.

In regard to the second problem, it is perfectly clear that the spawning reflex factor is not the same as the pituitary hormone, presumably LH, that elicits sperm release in male frogs.

In regard to the third problem, some further discussion is desirable since the negative results obtained by us conflict with other work that has been reported in the literature. Little work has been done on the effect of fish pituitaries on amphibians, and most of it concerns the effect on ovulation in the female frog or toad. Implantation into the dorsal lymph sac of either small or large numbers of pituitaries from the teleosts *Salvelinus namaycush, Perca flavescens* or *Stizostedion vitreum* (Creaser & Gorbman, 1936) and the elasmobranch *Squalus suckleyi* (Creaser & Gorbman, 1939) evoked no ovulatory response in *Rana pipiens.* Negative results were also obtained in female *Bufo arenarum* after repeated pituitary implants (Houssay *et al.,* 1929) and after the injection of saline extracts of glands from an unidentified species of fish (Houssay & Giusti, 1930). Similarly, ovulation was not induced in a species of *Rana* following the injection of extracts of glands from *Gadus merlangus* (Rostand, 1934). On the other hand, Wills, Riley & Stubbs (1933) obtained ovulation in the toad, *Bufo americanus,* after one to five daily injections of two to four pituitary glands of the gar, *Lepisosteus platysomus.* These authors also reported ovulation in *Rana pipiens* after three daily injections of four glands from the same species of fish.

Stroganov & Alpatov (1951) appear to be the only workers who have tested fish pituitaries using the gametokinetic response of male frogs. They obtained positive results within one hour in *Rana temporaria* and *R. ridibunda* using concentrations of 0.15 to 0.8 mgms. of acetone-dried pituitaries of the Russian sturgeon, *Acipenser güldenstädti.* Unspecified concentrations of this extract also evoked a positive response in *Rana esculenta.* These authors found that frogs were approximately three times more sensitive to injections of sturgeon pituitary than was the loach, *Misgurnus fossilis.*

The results of Stroganov & Alpatov stand in striking constrast to ours. Neither suspensions of acetone powders, at higher dosage levels than those employed by the Russian workers, nor extracts of a variety of purified or partially purified fish pituitary fractions, gave positive results with *Rana pipiens.* A weak and uncertain response was obtained even with an extract of whole frozen hake glands. Our experiments were made with pituitaries of the relatively specialized marine teleosts belonging to the family Gadidae, whereas Stroganov & Alpatov employed sturgeon glands. Creaser & Gorbman (1939) pointed out that the gars, *Lepisosteus,* are phylogenetically closer to the Amphibia than any of the other fishes whose pituitaries

had been used up to that time; the same relatively close relationship exists between the sturgeons and the Amphibia. It is obvious that phylogeny as well as species specificity may be involved.

That Stroganov & Alpatov found frogs to be more sensitive than fish for the assay of fish pituitary gonadotrophins, is also of interest in the light of the views of Creaser & Gorbman (1936, 1939). After extensive experimentation, these investigators concluded that interspecific difference in the gonadotrophic hormones was the factor responsible for the inactivity of fish gonadotrophins on female *R. pipiens*. This, they maintained, also made it necessary to use relatively high concentrations of mammalian hormones to evoke ovulation in amphibians, while homeo-transplants or implants were effective in much lower concentrations. However, our male frogs were very sensitive both to mammalian chorionic gonadotrophin and to LH. It is not possible to state whether or not hormone specificity is in part responsible for the negative results which we obtained with fish pituitary preparations. Even if hormone specificities are involved, however, there seems to be no close correlation with phylogenetic relationships.

Summary

1. Under the conditions of our experiments, the minimum dose of chorionic gonadotrophin (Antuitrin S, Parke Davis) that would elicit sperm release in *Rana pipiens* in one hour was 10 I. U. (= 0.04 mgms.). Armour's LH (80 to 85% homogeneous) was active at the same dosage level. The activity of a preparation of Armour's FSH was proportional to its LH content (three to four percent). Prolactin (Schering) was inactive.

2. A brei of frozen pituitary glands from the hake (*Urophycis tenuis*) occasionally elicited sperm release at a dosage level of two glands per frog (= ca. 4 mgms. dry weight).

3. Neither extracts of acetone-dried powders of hake, cod (*Gadus morhua*) or pollock (*Pollachius virens*), nor a variety of pituitary fractions derived from these species (A. E. Wilhelmi) showed any activity at doses of 4 mgms. or higher.

4. Neither fish nor beef pituitary fractions that were known to have spawning reflex activity when tested on the killifish, *Fundulus heteroclitus*, had any effect on male frogs.

5. It is concluded that the spawning reflex factor is not the same as the mammalian luteinizing hormone (LH).

6. The conflicting literature on the response of amphibians to fish pituitary gonadotrophins is discussed.

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